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Unusual Modification of Bacteriophage Mu DNA

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Bacteriophage Mu DNA was labeled after induction in the presence of [2-^{3}H]adenine or [8-^{3}H]adenine. Both Mu *mom*$^+$·*dam*$^+$ DNA and Mu *mom*$^-$·*dam*$^+$ DNA have similar N^6-methyladenine (MeAde) contents, as well as similar frequencies of MeAde nearest neighbors. Both DNAs are sensitive to in vitro cleavage by R·*Dpn*I but resistant to cleavage by R·*Dpn*II. These results indicate that the *mom*$^+$ protein does not alter the sequence specificity of the host *dam*$^+$ methylase to produce MeAde at new sites. However, we have discovered a new modified base, denoted A_x, in Mu *mom*$^+$·*dam*$^+$ DNA; approximately 15% of the adenine residues are modified to A_x. Although the precise nature of the modification is not yet defined, analysis by electrophoresis and chromatography indicates that the N^6-amino group is not the site of modification, and that the added moiety contains a free carboxyl group. A_x is not present in Mu *mom*$^+$·*dam*$^-$ or Mu *mom*$^-$·*dam*$^+$ phage DNA or in cellular DNA from uninduced Mu *mom*$^+$·*dam*$^+$ lysogens. These results suggest that expression of the *dam*$^+$ and *mom*$^+$ genes are required for the A_x modification and that this modification is responsible for protecting Mu DNA against certain restriction nucleases. Mu *mom*$^+$·*dam*$^-$ DNA and Mu *mom*$^-$·*dam*$^-$ DNA contain a very low level of MeAde (ca. 1 MeAde per 5,000 adenine residues). Since the only nearest neighbor to MeAde appears to be cytosine, we suggest that the methylated sequence is 5′...C-A*-C...3′ and that this methylation is mediated by the *Eco*K modification enzyme.

Escherichia coli bacteriophage Mu is unusual in that it is refractory to a variety of DNA restriction systems in vivo (20, 21) and in vitro (1, 11; R. Kahmann and D. Kamp, submitted for publication). This phenotype requires the activity of at least two genes, namely, the *mom*$^+$ gene of phage Mu and the host *E. coli dam*$^+$ gene (11, 20, 21), which controls the major DNA-adenine methylase activity in *E. coli* (14). For example, Mu *mom*$^+$ phage induced in *dam*$^-$ hosts are sensitive (efficiency of plating [EOP] $\leq 10^{-4}$) to P1 restriction, and Mu *mom*$^-$ mutants are also sensitive (EOP $\leq 10^{-4}$) to P1 restriction, even after growth in *dam*$^+$ hosts (20, 21). Furthermore, the resistance is also influenced by the manner in which the phage has been propagated; e.g., phage produced after thermal or spontaneous induction of lysogenic strains are resistant (EOP = 0.5 to 0.8), whereas lytic infection yields phage which are partially resistant (EOP = 0.01) to P1 restriction (20).

The above results suggest that the *mom*$^+$ gene product may interact with and alter the sequence specificity of the host *dam*$^+$ methylase. The *dam*$^+$ methylase (M·*Eco dam*) produces the sequence, 5′...G-A*-T-C...3′ (4, 7, 12), and the P1 modification methylase (M·*Eco* P1) produces 5′...A-G-A*-C-Py...3′ (12), where A* indicates N^6-methyladenine (MeAde). (Throughout this paper, the three-letter system of Smith and Nathans [19] is used to designate modification and restriction enzymes.) Thus, an alteration in M·*Eco dam* specificity could result in modification of P1 recognition sites. Although this possibility would explain the observed protection against P1 restriction, it is difficult to account for the protection against *Eco*K and *Eco*B restriction which have more complex sequences (13, 18; N. C. Kan, J. A. Lautenberger, M. E. Edgell, and C. A. Hutchison III, Abstr. Annu. Meet. Am. Soc. Microbiol. 1978, S145, p. 236). Nonetheless, the present investigation was designed to examine whether the DNA-adenine methylation pattern in phage Mu is affected by the *mom*$^+$ function. We show that, although the methylation pattern remains unchanged, approximately 15% of the adenine residues are modified to a new form, A_x. Both *mom*$^+$ and *dam*$^+$ genes are required for this modification.

MATERIALS AND METHODS

Phage and bacterial strains. Phage Mu*cIts*62 and Mu*cIts*62*momA* were obtained by thermal induction of lysogenic strains provided by A. Bukhari; these phages were used to prepare the lysogenic derivatives

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used in this study. *E. coli* 1100, F+ 1100, and F+ 1100(P1) are *endI thi*$^-$ r_K^- m_K^+ *dam*$^+$ (6); *E. coli dam*$^-$ is strain GM128 F+ *gal*$^-$ *lac*-2 r_K^+ m_K^+ *dam-4* (from M. Marinus); *E. coli dam*$^+$ is strain 1100 (from H. R. Revel).

Chemicals and media. Restriction nucleases R·*Dpn*I and R·*Dpn*II were generously provided by S. Lacks. Pancreatic DNase I (electrophoretically pure) was from Worthington Biochemicals Corp.; *E. coli* exonuclease I was the same preparation used in a previous study (7). H-broth contained (per liter): NaCl, 5 g; nutrient broth (Difco Laboratories), 8 g; peptone (Difco), 5 g; glucose, 1 g; thiamine hydrochloride, 1 mg. [2-^{3}H]adenine (15 Ci/mmol) and [8-^{3}H]-adenine (23 Ci/mmol) were from Amersham Corp., and 7-MeAde, 3-MeAde, and 1-MeAde were from Vega Biochemicals. MN-Polygram CEL 300 cellulose thin-layer sheets were from Brinkmann Instruments Inc.

Preparation of labeled phage DNA. Phage Mu DNA was labeled during growth in H-broth in the presence of [8-^{3}H]adenine or [2-^{3}H]adenine; all procedures were as described for phage λ (6), except that a 25-min heat induction period was used and the growth medium was H-broth. The phage were purified by differential centrifugation and equilibrium centrifugation in a CsCl density gradient (6); the DNA was purified and analyzed for MeAde content as described previously (5).

When unlabeled DNA was prepared, the phage (in crude lysates) were first precipitated in 2% (wt/vol) polyethylene glycol-0.5 M NaCl (22). The phage and DNA were then purified as described above.

Preparation of dinucleotides. Preparation of [2-^{3}H]adenine-labeled Mu DNA was as described above. The purified phage DNA was dialyzed against water and degraded enzymatically by successive treatments with pancreatic DNase I and *E. coli* exonuclease I (8). The resulting 5′ mononucleotides and 5′ dinucleotides were purified by DEAE-cellulose chromatography; the adenine-containing dinucleotides were purified by paper electrophoresis at pH 1.9 and 3.5. Under these conditions, the methylated and unmethylated nucleotides do not appreciably separate during electrophoresis (7, 8).

The isolated dinucleotides were dissolved in 1 N HCl and hydrolyzed for 1 h at 95°C, and the resulting purine bases were analyzed by descending chromatography (5).

RESULTS

Does *mom*$^+$ alter the specificity of the *dam*$^+$ methylase? The *E. coli dam*$^+$ methylase (M·*Eco dam*) recognizes the sequence 5′...G-A-T-C...3′ (4, 7, 12). It was possible that the *mom*$^+$ gene product alters the sequence specificity of M·*Eco dam* to methylate other sites and protect them against cleavage by restriction enzymes (e.g., the P1 restriction nuclease). If this notion were correct, then we might expect that the MeAde content or the methylation pattern or both would be different for Mu *mom*$^+$·*dam*$^+$ versus Mu *mom*$^-$·*dam*$^+$ DNA. Therefore, the MeAde content was determined for phage Mu grown in the presence of [2-^{3}H]adenine. Phage Mu is resistant to P1 restriction only when *mom*$^+$ and *dam*$^+$ genes are expressed (Table 1); this is in agreement with the results of Toussaint (20, 21). A small difference (ca. 20%) in MeAde contents was reproducibly observed for Mu *mom*$^+$·*dam*$^+$ DNA and Mu *mom*$^-$·*dam*$^+$ DNA; as will be discussed below, this apparent difference can be accounted for. Thus, *mom*$^+$ function does not appear to affect the overall MeAde content.

It should be noted that both Mu *mom*$^+$ and Mu *mom*$^-$ are almost devoid of MeAde after growth in a *dam*$^-$ host (Table 1); the remaining low level of MeAde appears to be due to methylation by the *E. coli* K modification methylase (to be discussed further below). We conclude that *mom*$^+$ does not control a DNA-adenine methylase that produces MeAde. In a separate experiment, we measured the ratio of [^{3}H]methylcytosine ([^{3}H]MeCyt) to [^{3}H]MeAde in DNA after the induction of phage Mu in medium containing [*methyl*-^{3}H]methionine. Mu *mom*$^-$·*dam*$^+$ DNA and Mu *mom*$^+$·*dam*$^+$ DNA exhibited similar ratios (0.49 and 0.36, respectively; data not shown); these results suggest that *mom*$^+$ does not control a phage DNA-cytosine methylase (if it does, then it methylates relatively few sites on Mu DNA).

To test whether *mom*$^+$ alters the sequence specificity of the *dam*$^+$ methylation, we followed two independent approaches. First, we examined whether the normal recognition site, G-A-T-C, is no longer methylated; this was tested by comparing the susceptibility of various Mu DNAs to in vitro cleavage by nucleases that cleave either

TABLE 1. *DNA methylation and plaque-forming ability of phage Mu*[a]

Phage	Plating efficiency on		MeAde (mol %)[b]
	F+ 1100	F+ 1100-(P1)	
Mu *mom*$^+$·*dam*$^+$	1.0	0.6	1.10 (1.02; 1.10; 1.10; 1.16)
Mu *mom*$^-$·*dam*$^+$	1.0	$\leq 10^{-4}$	0.92 (0.96; 0.89; 0.91; 0.90)
Mu *mom*$^+$·*dam*$^-$	1.0	$\leq 10^{-4}$	≤0.07 (≤0.07; ≤0.04; ≤0.08)
Mu *mom*$^-$·*dam*$^-$	1.0	$\leq 10^{-4}$	≤0.07

[a] Procedure for preparing labeled phage was as described in the text.

[b] Values represent counts per minute (cpm) of ^{3}H in MeAde/(^{3}H cpm in adenine + ^{3}H cpm in MeAde) × 100 and are the mean values obtained from several independently prepared labeled phage stocks; the values in parentheses show the range of values (variation was less than ±10% of the mean). The MeAde content of Mu *mom*$^-$·*dam*$^-$ was from a single determination; 1.00 mol % corresponds to approximately 190 MeAde residues per Mu DNA molecule.

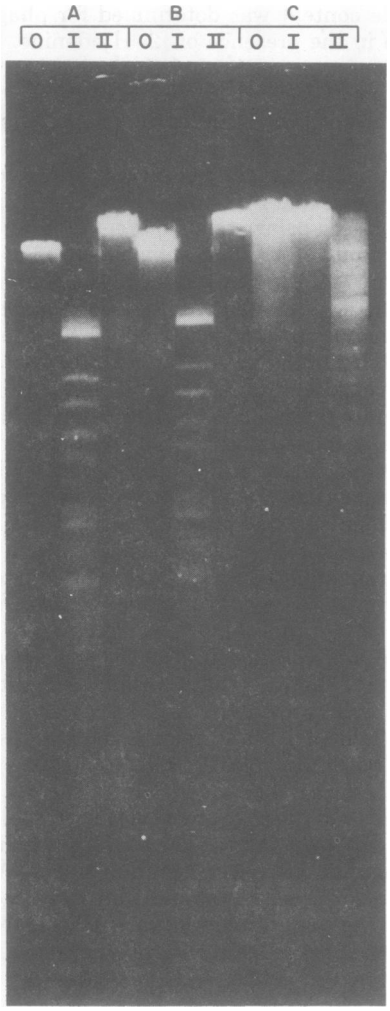

FIG. 1. *Agarose (tube) gel electrophoresis of Mu DNA treated with restriction nucleases R·DpnI and R·DpnII. Mu mom⁺·dam⁺, Mu mom⁻·dam⁺, or Mu mom⁺·dam⁻ DNA (ca. 1 μg) was incubated with 5 μl of R·DpnI or R·DpnII for 120 min at 37°C (in 50 mM Tris-hydrochloride, pH 7.6, 40 mM NaCl, 5 mM $MgCl_2$, and 0.1 mg of bovine serum albumin per ml in a total volume of 200 μl). The fragments were precipitated by addition of 1 μl of 4% (wt/vol) bovine serum albumin and 1 ml of cold 95% ethanol. After 20 min standing on ice, the fragments were harvested by centrifugation, dried under an air stream, and dissolved in 20 μl of 10 mM Tris-hydrochloride (pH 7.5) plus 5 μl of 20% sucrose, 25 mM Na_2-EDTA, and 125 μg of bromophenol blue per ml. The samples were applied to 1.2% agarose gels (0.6 by 13 cm) and subjected to electrophoresis at 120 V for 1.5 h. The running buffer was the same buffer used for the enzyme digestion. The gels were stained overnight at 4°C in 1 μg of ethidium bromide per ml; the gels were transilluminated with short-wavelength UV light and photographed through a red filter with Polaroid type 107 film. (A) Mu mom⁺·dam⁺ DNA; (B) Mu mom⁻·dam⁺ DNA; (C) Mu mom⁺·dam⁻ DNA. O, No enzyme added; I, R·DpnI; II, R·DpnII.*

(unmethylated) G-A-T-C or (methylated) G-A*-T-C sites. For example, R·*Dpn*II cleaves G-A-T-C, but not G-A*-T-C; in contrast, R·*Dpn*I has the reverse specificity (12). Thus, if *mom*⁺ alters M·*Eco dam* sequence specificity so that G-A-T-C is no longer methylated, then Mu *mom*⁺·*dam*⁺ and Mu *mom*⁻·*dam*⁺ should exhibit different sensitivity patterns; i.e., Mu *mom*⁺·*dam*⁺ DNA should behave like Mu *mom*⁺·*dam*⁻. As can be seen in Fig. 1, Mu *mom*⁺·*dam*⁺ DNA and Mu *mom*⁻·*dam*⁺ DNA have the same cleavage patterns; namely, they are both sensitive to R·*Dpn*I but resistant to R·*Dpn*II. In contrast, Mu *mom*⁺·*dam*⁻ was cleaved by R·*Dpn*II, but not by R·*Dpn*I. These results rule out the possibility that Mu *mom*⁺·*dam*⁺ DNA contains both unmethylated and methylated (or hybrid) G-A-T-C sites; rather, it appears that the G-A-T-C sites are all protected (methylated) and must be G-A*-T-C. We conclude that *mom*⁺ does not alter M·*Eco dam* so that G-A-T-C sites are no longer recognized.

These results still leave open the possibility that M·*Eco dam* methylation occurs at G-A-Py-C, rather than G-A-T-C. This notion could be experimentally tested by analyzing the nearest neighbors to MeAde; operationally this is accomplished by determining the MeAde content of purified dinucleotides. To do this, we prepared phage DNA labeled after induction in the presence of [2-^{3}H]adenine; after enzymatic degradation, by successive treatment with pancreatic DNase I and *E. coli* exonuclease I, ^{3}H-labeled dinucleotides were purified by DEAE-cellulose chromatography and paper electrophoresis (8). After hydrolysis in HCl, the liberated purine bases were analyzed for [^{3}H]Ade and [^{3}H]-MeAde by descending paper chromatography; Table 2 lists the MeAde content of each dinucleotide. Since the major methylase activity in *E. coli* is M·*Eco dam* (Table 1) (14), we would expect that both the (G,A) and (A,T) dinucleotides would contain high levels of MeAde (derived from G-A*-T-C). It is evident that Mu *mom*⁺·*dam*⁺ DNA and Mu *mom*⁻·*dam*⁺ DNA do, in fact, contain MeAde in the (G,A) and (A,T) dinucleotides. If *mom*⁺ altered M·*Eco dam* specificity to methylate G-A-Py-C, then we would also expect to find a high level of MeAde in (A,C) from Mu *mom*⁺·*dam*⁺ DNA, but not from Mu *mom*⁻·*dam*⁺ DNA or Mu *mom*⁺·*dam*⁻ DNA. All three phage DNAs contain a low level of MeAde in (A,C); the only MeAde in Mu *mom*⁺·*dam*⁻ DNA is in this dinucleotide (ca. 1

TABLE 2. *Distribution of MeAde in adenine-containing dinucleotides from Mu DNA*[a]

Phage DNA	MeAde in dinucleotide (mol %)[b]			
	(A,A)	(A,C)	(A,G)	(A,T)
Mu *mom*$^+$·*dam*$^+$	<0.002	0.10	2.30	1.17
Mu *mom*$^-$·*dam*$^+$	<0.002	0.09	1.51	1.10
Mu *mom*$^+$·*dam*$^-$	<0.005[c]	0.14[c]	<0.002[c]	<0.002[c]

[a] Procedures were as described in the text.

[b] See footnote *b* to Table 1. The values presented are the mean values of two independent labeled DNA preparations; in some instances, replicate chromatographic analyses were carried out. The different MeAde contents of (G,A) and (A,T) may be due to differences in the recovery of the dinucleotides, as well as to specificity of DNase I cleavage. If we assume that all four dinucleotides are present in equal frequency, the average mole percent MeAde in dinucleotides is similar to that in total DNA (Table 1). The mole percent MeAde in the mononucleotide fraction was also determined (data not shown); the results were similar to those observed in total DNA (Table 1).

[c] Based on a single determination.

MeAde residue per 1,000 adenine residues) (Table 2). Since all three DNAs carry *Eco*K modification, we propose that this methylation is due to M·*Eco*K activity and that the *Eco*K-modified sequence includes 5′ C-A*-C 3′; this is consistent with the observation that the K recognition site contains G-C-A-C (Kan et al., Abstr. Annu. Meet. Am. Soc. Microbiol. 1978, S145, p. 236). In conclusion, it appears that the M·*Eco dam* sequence specificity is not altered by the *mom*$^+$ function.

***Mom*$^+$ controls a new modification of adenine residues.** The results of the previous section indicated that MeAde and MeCyt modification levels were not affected by *mom*$^+$. In view of these results, it seemed reasonable to consider the possibility that adenine or guanine residues are methylated at the N-1, N-3, or N-7 position. In this regard, 7-methylguanine has been reported as a minor component of *Shigella* phage DDV1 DNA (17). Therefore, DNA was purified from various Mu phages labeled after induction in the presence of [8-^{3}H]adenine. The DNA was hydrolyzed in acid, and the purine bases were analyzed by a variety of methods. Figure 2 shows the profile obtained after cellulose thin-layer chromatography. Mu *mom*$^+$·*dam*$^+$ DNA exhibited a large peak of ^{3}H radioactivity between 7-MeAde and N^6-MeAde (in other analyses, this peak chromatographed closer to N^6-MeAde). This material is denoted A_x to indicate that it is an adenine derivative; this is supported by the fact that DNA labeled with [2-^{3}H]adenine also contains A_x. It is evident in Fig. 2 that Mu *mom*$^-$·*dam*$^+$ DNA does not contain A_x. In addition, separate experiments showed that Mu *mom*$^+$·*dam*$^-$ DNA is also devoid of A_x. Therefore, the presence of A_x is specific for Mu *mom*$^+$·*dam*$^+$ DNA, suggesting that *mom*$^+$ and *dam*$^+$ gene functions are required for A_x modification. We also analyzed the DNA from uninduced lysogenic cells labeled during growth in [8-^{3}H]adenine. We observed no A_x in DNA from lysogens of Mu *mom*$^-$·*dam*$^+$ or Mu *mom*$^+$·*dam*$^+$ (data not shown). This is consistent with the data of Toussaint (20), who concluded that *mom*$^+$ function is not expressed in uninduced lysogenic cells.

It is also clear in Fig. 2 that A_x represents a significant fraction of the adenine bases; approximately 15% of the ^{3}H label is in A_x. When the same DNA hydrolysates were analyzed for [^{3}H]adenine and [^{3}H]MeAde by our standard descending paper chromatography system (86% *n*-butanol with an NH_3 atmosphere), we observed the expected 1% MeAde for both DNAs.

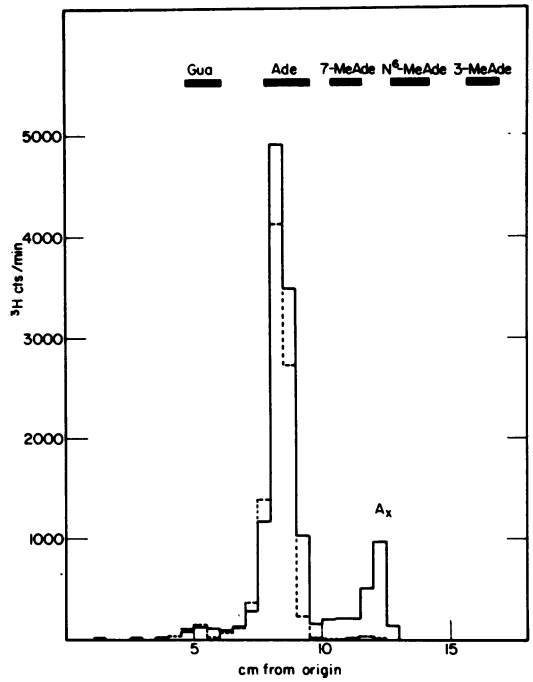

FIG. 2. *Cellulose thin-layer chromatography of Mu mom*$^+$·*dam*$^+$ *and Mu mom*$^-$·*dam*$^+$ *DNA hydrolysates. Phage DNA was labeled in the presence of [8-*^{3}*H]adenine after prophage induction. The purified DNA was hydrolyzed in 1 N HCl, and portions were subjected to ascending chromatography on Brinkmann MN Polygram CEL-300 sheets in* CH_3OH*–concentrated HCl–water (70:20:10) for 8.5 h at 20°C. After drying in air, the positions of authentic markers were located under UV light. Strips (0.5 by 2.0 cm) were cut (from the origin along the direction of chromatography) and placed in scintillation vials containing 0.5 ml of water. Fluor was added, and the* ^{3}*H radioactivity in each sample was determined. Mu mom*$^+$·*dam*$^+$ *(——); Mu mom*$^-$·*dam*$^+$ *DNA (-----).*

In view of the high chromatographic mobilities of adenine and MeAde, we had generally ignored the region from the origin to guanine (ca. 6 cm from the origin). However, when we examined this region we found a significant peak of ^3H radioactivity near the origin (Fig. 3) that was present only with Mu *mom*$^+$·*dam*$^+$. When this was excised, eluted, and subjected to thin-layer chromatography, the ^3H radioactivity migrated to the position of A_x. Thus, we had been overlooking A_x in our previous analyses (summarized in Tables 1 and 2) of Mu *mom*$^+$·*dam*$^+$ DNA. In fact, when one now takes A_x into account, the moles percent MeAde for Mu *mom*$^+$·*dam*$^+$ and Mu *mom*$^-$·*dam*$^+$ are identical.

It is evident from comparisons with authentic markers of adenine derivatives that A_x is not hypoxanthine, N^6-MeAde, 1-MeAde, 3-MeAde, or 7-MeAde. It appears that the modification involves the addition of a substituent containing an acidic group. For example, at pH 3.5, A_x has a slight positive charge and it coelectrophoreses with hypoxanthine (Fig. 4a); at pH 8.0, A_x has a high negative charge and it migrates slightly slower than 5′-dAMP (Fig. 5b). At pH 1.9, A_x has a high positive charge, and it migrates faster than hypoxanthine (Fig. 4b). Since the pK_1 of a nucleotide phosphate is less than 1, it is likely that the acidic function is a carboxyl group and not a phosphate. The modification does not appear to occur at the exocyclic N^6-NH_2 group. This was shown as follows: [8-^{3}H]adenine-labeled A_x was purified by paper chromatography (as in Fig. 3) and incubated in the presence of nitrous acid (under conditions where marker MeAde was completely deaminated to hypoxanthine). Electrophoretic analysis (Fig. 4) revealed that deamination of A_x does not produce hypoxanthine; at pH 3.5 and 1.9 the product had a different electrophoretic mobility than hypoxanthine. The electrophoretic properties of deaminated A_x are also consistent with the presence of a carboxyl group. Finally, A_x was subjected to acid-catalyzed esterification with methanol. At pH 3.5, the A_x ester had a higher positive charge than A_x; at pH 8.0, the A_x ester was uncharged, whereas A_x was negatively charged (Fig. 5). These results confirm the presence of a free carboxyl group on A_x.

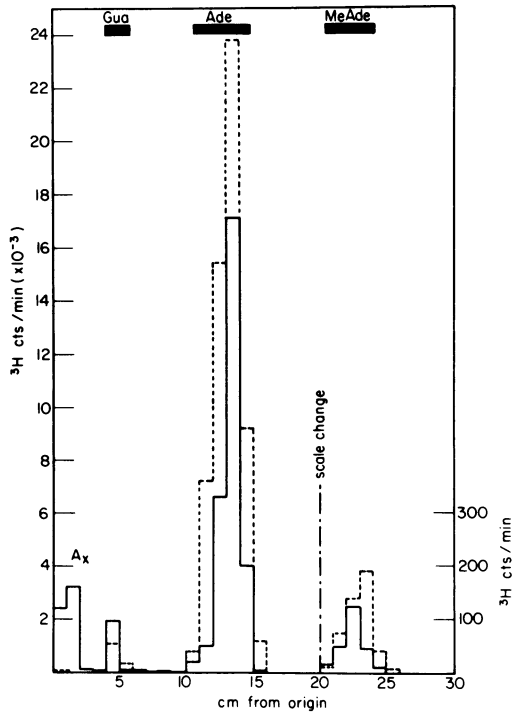

FIG. 3. *Paper chromatography of [8-^{3}H]adenine-labeled Mu DNA hydrolysates. Portions of hydrolyzed Mu DNA (see legend to Fig. 2) were subjected to descending (Whatman 1) paper chromatography in 86% butanol (NH_3 atmosphere) for 17 h at 20°C. Authentic markers (indicated by solid bars) were located under UV light; the paper was cut into strips (1 by 2 cm) and placed in scintillation vials containing 0.5 ml of water. Fluor was added, and the ^3H radioactivity was determined. The figure contains superimposed profiles for Mu mom*$^+$·*dam*$^+$ (——) *and Mu mom*$^-$·*dam*$^+$ (-----). *Mu mom*$^+$·*dam*$^-$ *was also analyzed, but to avoid confusion, it is not included in the figure. However, no ^3H radioactivity was found near the origin.*

DISCUSSION

The data presented in this communication appear to exclude the possibility that the *mom*$^+$ protein alters M·*Eco dam* sequence specificity. This conclusion follows from the observations that the MeAde content (Table 1), nearest neighbors (Table 2), and methylation of G-A-T-C sequences (Fig. 1) are similar for Mu *mom*$^+$ and Mu *mom*$^-$. However, we have discovered that in Mu *mom*$^+$·*dam*$^+$ phage DNA a significant fraction of the adenine residues (ca. 15%) is modified to a new form, A_x. Since Mu *mom*$^-$·*dam*$^+$ and Mu *mom*$^+$·*dam*$^-$ do not contain A_x, it appears that *dam*$^+$ and *mom*$^+$ gene expression are required for A_x production. Moreover, bacterial DNA from uninduced cells lysogenic for Mu *mom*$^+$ do not contain A_x; this is consistent with the conclusion that *mom*$^+$ is not expressed in uninduced lysogens (20). It should be noted that a previous investigation did not reveal A_x in Mu DNA (15); however, in this study the phage were grown lytically, and under these conditions *mom*$^+$ is poorly expressed (20).

The evidence that A_x is an adenine, and not a guanine, derivative stems from the nature of the

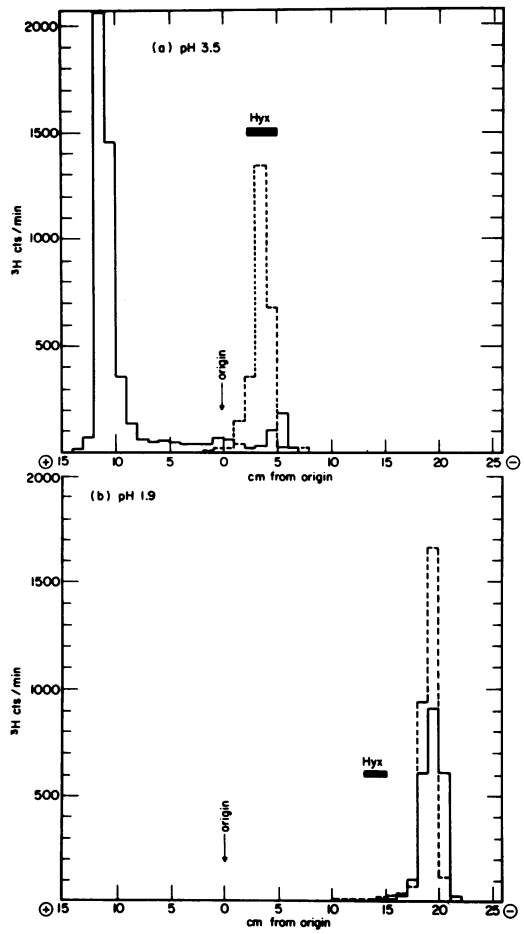

FIG. 4. *Paper electrophoresis analysis of [8-^{3}H]adenine-labeled A_x before and after deamination with nitrous acid. (a) [8-^{3}H]adenine-labeled A_x was purified by paper chromatography (Fig. 3) of a Mu mom$^+$·dam$^+$ DNA hydrolysate. A portion was suspended in 10 μl of 2 M $NaNO_2$ + 10 μl 0.50 M sodium acetate (pH 3.9) + 2 μl of MeAde (2 mg/ml). The mixture was incubated for 15 h at 20°C, applied to Whatman 3 MM strips (2 by 58 cm; origin was 20 cm from anode), and subjected to flat-plate (Savant Instruments, Inc.) electrophoresis for 2 h at 2 kV in 0.05 M NH_4COOH (pH 3.5). The carrier MeAde was completely converted to hypoxanthine (Hyx), as shown by the solid bar. The figure shows, superimposed, the results of parallel analyses of untreated A_x (-----) and deaminated A_x (——). (b) Deaminated A_x was purified after electrophoresis at pH 3.5 (as shown above) and analyzed by electrophoresis at pH 1.9 (2.5% HCOOH, 8.7% CH_3COOH) for 2 h at 2 kV (the origin was 20 cm from the anode). The figure shows, superimposed, the results of parallel analyses of untreated A_x (-----) and deaminated A_x (——).*

isotopic precursors. Both [8-^{3}H]adenine and [2-^{3}H]adenine incorporation produce ^{3}H-labeled A_x; since metabolic conversion of [2-^{3}H]adenine to guanine would remove the ^{3}H label, it is clear that A_x has to be a modified adenine. A_x does not appear to be a nucleoside or nucleotide, since it is resistant to perchloric acid hydrolysis and alkaline phosphatase. The chromatographic and electrophoretic properties of A_x indicate that the added substituent contains a free carboxyl group (Fig. 4 and 5). The modification does not occur at the exocyclic N^6-NH_2 group, as revealed by nitrous acid deamination (Fig. 4). Thus, it appears that the modification must occur at the N-1, N-3, or N-7 position. Further studies are planned to elucidate the structure of A_x.

It is interesting to note that the MeAde content appears to be unaffected by the presence of A_x; i.e., after correction for the adenine → A_x conversion, both Mu *mom*$^+$ and Mu *mom*$^-$ have virtually identical MeAde contents. Thus, *mom*$^+$ does not act by modifying MeAde-containing sequences. However, the *mom*$^+$ modification does appear to have sequence specificity (and, therefore, must occur as a post-DNA-replication event). This has been clearly demonstrated by the fact that Mu *mom*$^+$·*dam*$^+$ DNA is resistant to in vitro cleavage by a select group of restriction nucleases (Kahmann and Kamp, submitted for publication). The recognition sites for these enzymes all contain adenine (a necessary but not sufficient condition for resistance), which could be rendered resistant by modification to A_x. Kahmann and Kamp (submitted for publication) suggested that the recognition site for the *mom*$^+$ modification is the pentanucleotide sequence, 5′ ... $\binom{C}{G}$-A-$\binom{G}{C}$-N-Py ... 3′. In this regard, we have observed that there are only two dinucleotides containing A_x; electrophoretic analyses suggest that one of them is (A_x, G) and the other is (A_x, C) (manuscript in preparation).

For the present, we have not gained any insight into the nature of the host *dam*$^+$ methylase role in the *mom*$^+$ modification; e.g., we found no evidence for an alteration in the methylase specificity/methylation pattern. It had been suggested earlier (1, 20) that inversion of the Mu G segment (2, 3, 9) may be obligatory for *mom*$^+$ expression. If so, the requirement for *dam*$^+$ methylation might be due to a role in the G inversion process. However, restriction nuclease digestion and DNA heteroduplex analyses indicate that *dam*$^+$ methylation is *not* required for production of the G-inverted orientation (unpublished data).

Finally, the analysis of MeAde content in purified dinucleotides (Table 2) showed that the low level of MeAde in Mu *mom*$^+$·*dam*$^-$ is not due to M·*Eco dam* methylation, since the MeAde was exclusively in the (A,C) dinucleotide. The DNA from Mu *mom*$^+$·*dam*$^+$, Mu

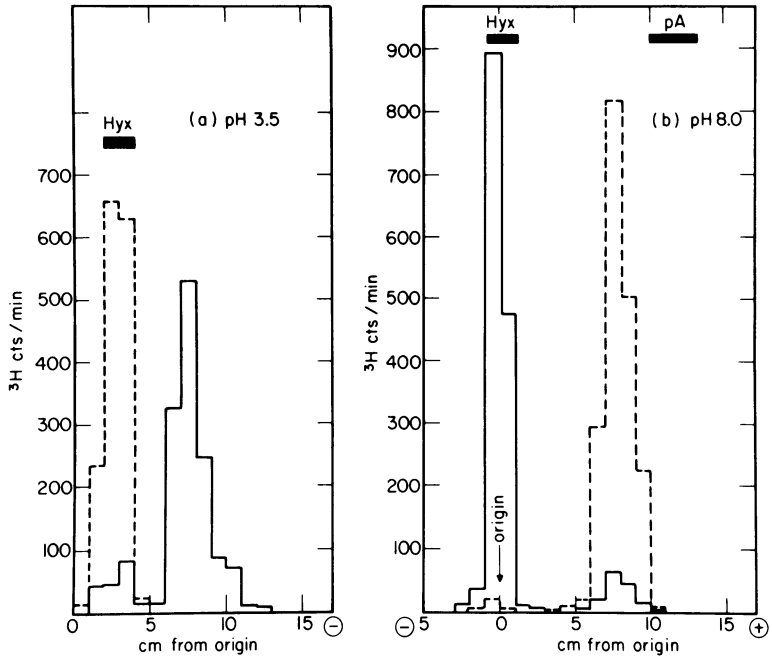

FIG. 5. *Paper electrophoresis analysis of [8-^{3}H]adenine-labeled A_x before and after esterification with methanol. [8-^{3}H]adenine-labeled A_x (see Fig. 4) was suspended in anhydrous methanol and added to a mixture of anhydrous methanol-acetyl chloride (10:1, vol/vol). The solution was heated in an oil bath for 20 h at 78°C. The methanol was refluxed with the aid of a water-cooled condenser. The methanol was evaporated under a stream of nitrogen, and the A_x ester was suspended in water and analyzed by paper electrophoresis as described in the legend to Fig. 4. Authentic markers of hypoxanthine (Hyx) and 5'-dAMP (pA) were included in the same analysis and are indicated by the solid bars. (a) Electrophoresis for 2 h at 2 kV in 0.05 M NH_4COOH (pH 3.5); origin was 20 cm from the anode. (b) Electrophoresis for 1 h at 2 kV in 0.1 M Tris-hydrochloride (pH 8.0); origin was 20 cm from the cathode. A_x ester (——); untreated A_x (----).*

mom$^-$·*dam*$^+$, and Mu *mom*$^+$·*dam*$^-$ all contain a similar low level of MeAde in the (A,C) dinucleotide [this rules out (A,C) methylation by the *mom*$^+$ protein]. Since all three DNAs carry *Eco*K modification, we propose that (A,C) methylation is due to M·*Eco*K, and that the methylated *Eco*K site contains 5′ C-A*-C 3′. This is consistent with the sequence data of Kan et al. (Abstr. Annu. Meet. Am. Soc. Microbiol., 1978, S145, p. 236), who observed G-C-A-C in two K recognition sites. We calculate that there is 1 MeAde per 1,000 adenine residues in the (A,C) dinucleotide; thus, in Mu *mom*$^+$·*dam*$^-$ DNA there is approximately 1 MeAde residue per 5,000 adenine residues, or approximately 5 MeAde residues per Mu DNA molecule. It is interesting to note that there are only 5 *Eco*K sites per λ DNA (16), which is somewhat larger than Mu DNA.

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